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EFFECT OF DETERGENTS AND SODIUM FLUORIDE ON THE ENZYME ACTIVITIES OF THE RAT LIVER PLASMA MEMBRANE

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SUMMARY

The effects of ionic and non-ionic detergents on the absorbance and enzyme activities of the rat liver plasma membrane have been studied. The absorbance (turbidity) *versus* detergent concentration titration curve was characteristic and non-linear for each detergent. At low detergent concentration (up to 0.025 %) the non-ionic detergents were either more effective or equally effective as the anionic detergents in solubilizing the membrane but at higher detergent concentration (0.04–0.10 %), the anionic detergents were more effective.

Sodium dodecyl sulfate at 0.1 % strongly inhibited ATPase (EC 3.6.1.3) and 5′-nucleotidase activities (EC 3.1.3.5) whereas deoxycholate gave a slight stimulation of ATPase and a moderate stimulation of 5′-nucleotidase. Triton X-100 had no effect on ATPase but stimulated 5′-nucleotidase. Lubrol-PX gave a moderate stimulation of both these enzymes.

0.1 % sodium dodecyl sulfate stimulated adenylate cyclase (formerly known as adenyl cyclase) activity in crude membranes and in purified membranes prepared either by the method of Neville or the method of Ray. However, NaF-stimulated adenylate cyclase in crude membranes or in membranes prepared by the method of Neville[22] but inhibited adenylate cyclase in membranes made by the method of Ray[21].

The effect of sodium dodecyl sulfate and NaF on ATP metabolism by the membrane showed these agents inhibit the conversion of ATP to ADP, AMP and adenosine but the effects are dependent on the concentration of membrane. At low membrane concentration, ATPase is the dominant enzyme but at high membrane concentration 5′-nucleotidase plays an important role in the overall metabolism of ATP.

These studies demonstrate a complex interaction of detergents with membrane-bound enzymes and a complex system for nucleotide degradation in the plasma membrane.

INTRODUCTION

The relationship between membrane structure and the activity of membrane-bound enzymes is an area of current research interest in membrane biochemistry[1–6].

The interaction of detergents with proteins, lipoproteins and membranes can yield useful information and may have practical applications. For example, the polyacrylamide gel electrophoresis of sodium dodecyl sulfate treated proteins can reveal the sub-unit nature of proteins and is commonly used for the determination of the molecular weight of the subunits[7,8]. Detergents have been used to solubilize lipoproteins and membranes[6,9–12]. Detergent treated membranes yield many protein fractions when subjected to gel electrophoresis[13–16].

Some enzymes which are associated with membranes lose much of their activity when treated with certain detergents. ATPase and 5′-nucleotidase are two such enzymes[17–19]. Other membrane bound enzymes are not inhibited by detergent treatment. Dallner[6] has studied the effect of detergent concentration on the activity of certain microsomal enzymes. These studies have shown that the concentration of detergent *versus* enzyme activity profiles vary considerably with different enzymes. Emmelot and Bos[20] have found (Na^+, K^+)-ATPase and 5′-nucleotidase of rat liver plasma membranes to be stimulated by deoxycholate and Lubrol.

In order to correlate membrane alteration by detergent with enzyme activity the absorbances (in this case a measure of turbidity by light scattering) of the membranes were measured at varying concentrations of detergents with and without sonication. Different types of anionic and non-ionic detergents were tested. The activities of three enzymes were studied, these being Mg^{2+}-ATPase, 5′-nucleotidase and adenylate cyclase. The results of these studies are presented in this paper.

METHODS AND MATERIALS

Rat liver plasma membranes were prepared by the methods of Ray[21] and Neville[22]. The membranes made by the Neville method excluded steps 12–15 and thus represent the partially purified membrane. Membrane adenylate cyclase activity was assayed in a system containing 50–100 μg membrane protein, 0.5 mM [^{14}C]-ATP, (0.4-0.6 μCi) [8-^{14}C]adenosine-5′-triphosphate tetrasodium salt, spec. act. 1.02 mCi/mmole (New England Nuclear and Amersham-Searle), 1 mM $MgCl_2$, and 0.05 M Tris buffer, pH 7.5, in a final volume of 0.5 ml. This mixture was incubated for 15 min at 37 °C, and the reaction stopped by immersing the tubes in boiling water for 3 min. ATP, ADP, AMP, adenosine, adenine, hypoxanthine and inosine were separated from cyclic AMP by two dimensional chromatography on Whatman No. 3 paper: Solvent 1: isopropanol–concentrated NH_4OH–water (7:2:1, v/v); Solvent 2: isopropranol–formic acid–water (70:5:30, v/v). ATP and ADP were further separated on Whatman No. 1 filter paper using isobutyric acid–concentrated NH_4OH–water (66:1:33, v/v). 5–15 μg of each nucleotide, (nucleoside or base) were also spotted on the chromatogram to allow for visualization of the spots under ultraviolet light. The separated nucleotides, nucleosides and base were cut out of the chromatogram and counted in a Packard Liquid Scintillation counter in 10 ml of Bray's cocktail[23].

Mg^{2+}-ATPase and 5′-nucleotidase were assayed in the following system: 0.5 M Tris buffer, pH 7.5, 10 mM $MgCl_2$, 40–60 μg membrane protein, and either 10 mM adenosine 5′-triphosphate or 10 mM adenosine 5′-monophosphate, in a total volume of 1 ml. The reaction mixture was incubated for 20 min at 37 °C, then stopped by adding 1 ml of 10 % trichloroacetic acid. ATPase and 5′-nucleotidase activity are

reported in μmoles P_i/mg membrane protein. These assays are modified after Ray[21]. Phosphorus was determined by the method as previously described[24].

Adenylate cyclase, Mg^{2+}-ATPase, and 5'-nucleotidase activities were also assayed in the presence of 0.05 % and 0.1 % Lubrol-PX, sodium deoxycholate, sodium dodecyl sulfate or Triton X-100.

Solubilization of the membranes in 50 mM Tris buffer, pH 7.5, containing detergent was determined by following the change in absorbance (turbidity by light scattering) at 525 nm with a Beckman DU spectrophotometer.

Nucleotides were obtained from PL Biochemicals. Sodium dodecyl sulfate obtained from Fisher Scientific Co., and sodium deoxycholate from Sigma Chemicals. Triton X-100 was a gift from Rohm and Haas, Philadelphia, Pa., and Lubrol-PX was a gift from ICI America Inc., Stamford, Conn. The ionic detergents Alipal Co. 433 and 436, and Igepal CO 630 were gift samples from GAF Corp. Tween-20 was obtained from Atlas Corp.

RESULTS

The titration curves of the absorbance (turbidity) of the membrane at varying concentrations of detergents are shown in Fig. 1. Each detergent gave a characteristic non-linear curve. Over the relatively low concentration range studied, sodium dodecyl sulfate had the greatest solubilizing effect on the membrane but only above 0.025 %. Triton and Lubrol, the non-ionic detergents, were either more effective or equally effective as the anionic detergents sodium dodecyl sulfate and deoxycholate at low concentrations, but at the higher concentrations (0.04–0.10 %) sodium dodecyl sulfate was the most effective solubilizing agent.

When membranes were treated with detergents and then sonicated, all three detergents gave approximately the same degree of membrane solubilization, but

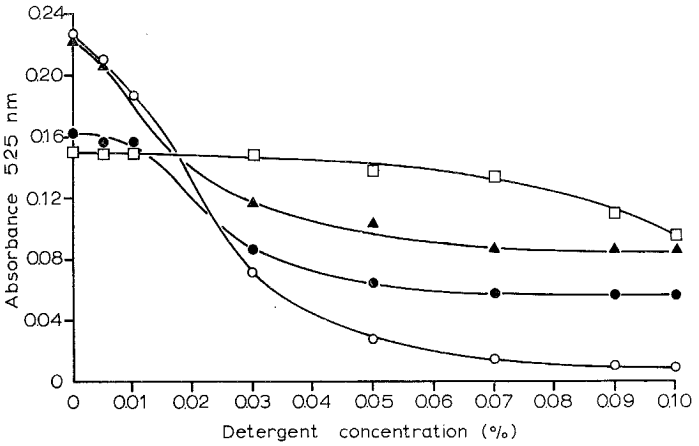

Fig. 1. The effect of low concentration of sodium dodecyl sulfate (○—○); deoxycholate (□—□); Triton (●—●) and Lubrol (▲—▲) on the solubilization of the plasma membranes. Plasma membranes (approx. 10 mg) were suspended in 20 ml of 0.05 M Tris buffer, pH 7.5. Aliquots were treated with detergents at the concentrations shown in the figure. The membranes were mixed on a vortex mixer and the absorbance read after 5 min at 525 nm. The results shown in Figs 1 to 4 are examples of experiments repeated several times.

only at concentrations above 0.05 % (Fig. 2). It is thus apparent that with the aid of sonication, Triton and deoxycholate can disrupt the membrane as effectively as can sodium dodecyl sulfate. All three detergents give fairly sharp inflection profiles, but at different concentration ranges. At these inflection regions the change in turbidity produced per unit amount of detergent is most pronounced.

In Fig. 3 the effects of sodium dodecyl sulfate, deoxycholate, Lubrol and Triton at the higher concentration range of 0.1–0.8 % are shown. Sodium dodecyl sulfate and deoxycholate (anionic detergents) are more effective solubilizing agents

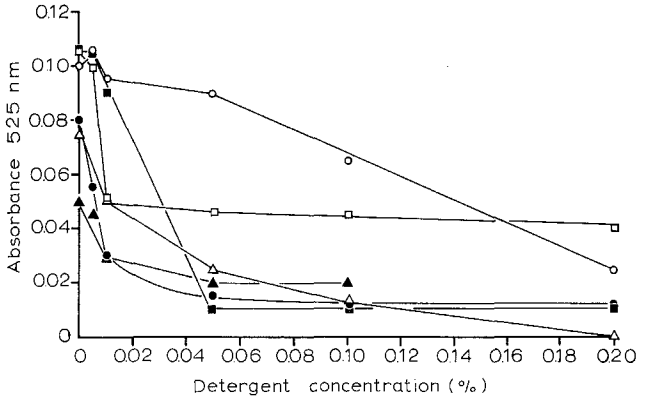

Fig. 2. The effect of sonication on detergent treated membranes. The membranes were treated as in Fig. 1. Aliquots were also sonicated with a Bronson Sonifier Model LS 75 for 45 s at room temperature. The absorbance was read after 5 min at 525 nm. Triton (□—□); Triton-sonicated (▲—▲); sodium dodecyl sulfate (■—■), sodium dodecyl sulfate-sonicated (●—●); deoxycholate (○—○); deoxycholate-sonicated (△—△).

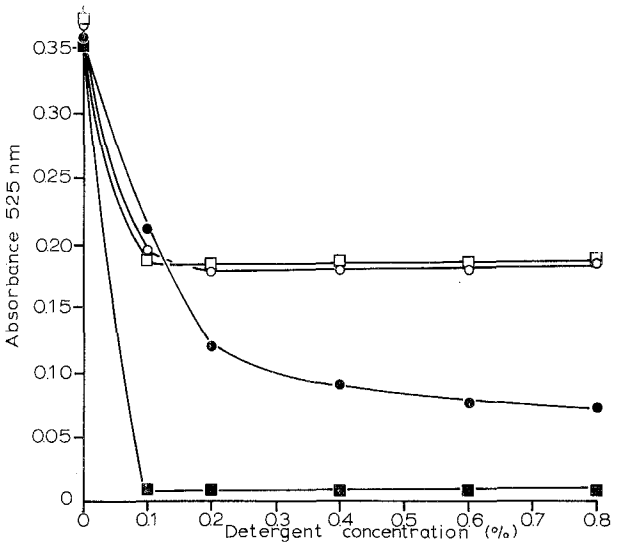

Fig. 3. The effect of high concentrations of sodium dodecyl sulfate (■—■); deoxycholate (●—●); Triton (□—□) and Lubrol (○—○) on the solubilization of the plasma membranes. The membranes were treated as in Fig. 1 except the detergents were studied over a higher concentration range.

than Lubrol and Triton (non-anionic detergents). Thus at high detergent concentrations where the full expression of the detergent is possible, negatively charged detergents are more potent in fragmenting the membrane. This may in large part be due to these detergents producing fragments having high negative charge.

Several other detergents were tested. The results in Fig. 4 show that the non-ionic detergent Tween-20 had no appreciable effect on solubilizing the membrane but the anionic detergents Igepal and Alipal were effective solubilizing agents. It is of interest that some detergents at very low concentrations (below 0.02 %) increased the turbidity of the membranes. This suggests that a detergent–membrane complex first forms and only at higher detergent concentration is the membrane fragmented.

In order to see what size fragments were obtained by the action of detergents on the membrane, the detergent treated membranes were fractionated on Sephadex G-200. Untreated membranes do not pass through the column. The column elution profiles for membranes treated with sodium dodecyl sulfate, deoxycholate and Lubrol (at 0.1–0.15 % concentration) are shown in Fig. 5. In the case of sodium dodecyl sulfate and deoxycholate the major peak emerges just past the void volume (about 8 ml) indicating fragments of large size. Although sodium dodecyl sulfate decreased the turbidity of the membrane more than did deoxycholate and Lubrol (Fig. 1), the column fractionation indicated that the sodium dodecyl sulfate fragments were larger in size than the deoxycholate or Lubrol fragments. Therefore the decrease in turbidity is not necessarily correlated with small size membrane particles, but suggests that sodium dodecyl sulfate also alters the structure of the membrane in such a way that its optical properties (molecular arrangement) are altered.

The effect of detergents on the membrane bound enzymes was investigated (Table I). At a concentration of 0.1 %, only sodium dodecyl sulfate inhibited the Mg^{2+}-ATPase and 5′-nucleotidase activities. Deoxycholate gave a slight stimulation of ATPase and a moderate stimulation of 5′-nucleotidase. Triton had no effect on ATPase but stimulated 5′-nucleotidase. Lubrol gave a moderate stimulation of both

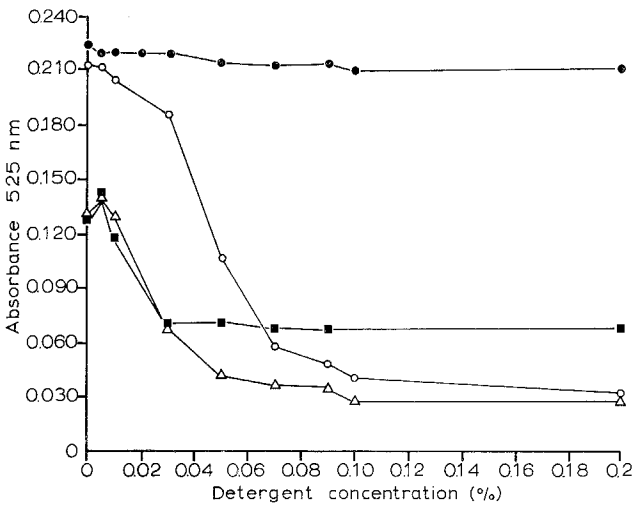

Fig. 4. The effect of Tween 20 (●—●); Igepal CO-630 (■—■); Alipal CO-433 (○—○) and Alipal CO-436 (△—△) on the solubilization of the plasma membranes. The membranes were treated as in Fig. 1 except different detergents were examined.

enzymes. These results showed that a direct correlation of the turbidity effects of the detergents with the enzyme effects does not exist. At 0.1 % detergent concentration the membranes had undergone extensive alteration as evidenced by the large decrease in turbidity and by the column fractionation profiles. Although the membranes were extensively altered by the detergents, some inhibited ATPase and 5′-nucleotidase and others stimulated the activity of these enzymes.

The effects of sodium dodecyl sulfate and NaF on the overall metabolism of ATP by the membrane are given in Fig. 6. It can be seen that sodium dodecyl sulfate

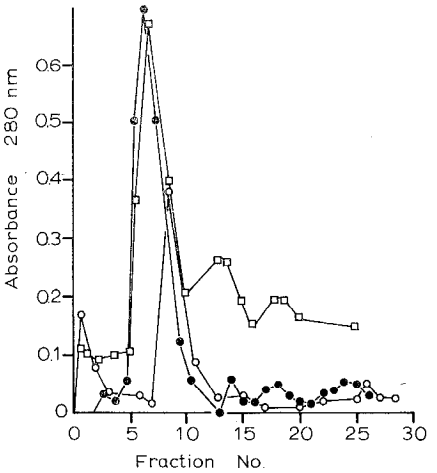

Fig. 5. Column chromatographic separation of detergent treated plasma membranes on Sephadex G-200. Membranes (approx. 4–5 mg) in 0.05 M Tris buffer, pH 7.5, containing detergents at the concentration shown, were fractionated on a column of Sephadex G-200 measuring 1.8 cm × 16 cm. The elution was carried out in 0.05 M Tris buffer, pH 7.5. 2-ml fractions were collected. The protein was determined by measuring the absorbance at 280 nm. 0.15% deoxycholate (□—□); Lubrol (○—○) and sodium dodecyl sulfate (●—●).

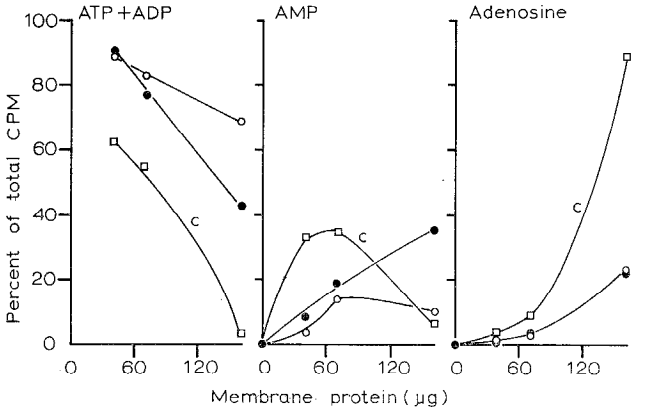

Fig. 6. The effect of sodium dodecyl sulfate and NaF on the metabolism of ATP in isolated plasma membranes. Membranes were incubated with and without 0.1% sodium dodecyl sulfate and 10 mM NaF. The incubation was carried out in 0.05 M Tris buffer, pH 7.5. The membranes were studied at varying concentrations as shown in the figure. After 20 min incubation at 37 °C aliquots of the incubation medium were removed for analysis of ATP, ADP, AMP and adenosine as explained in the text. Sodium dodecyl sulfate (○—○); NaF (●—●) and Control (□—□).

and NaF inhibit the conversion of ATP to ADP, AMP and adenosine. The overall effects are dependent on the concentration of membrane. At low membrane concentration, the ATPase is the dominant enzyme but at high membrane concentration 5'-nucleotidase plays an important role. At high membrane concentration adenosine is the major end product. Sodium dodecyl sulfate and NaF inhibit formation of adenosine by inhibiting both ATPase and 5'-nucleotidase. Sodium dodecyl sulfate has a greater effect on ADP and AMP production than does NaF.

In order to study the effect of an ATP regenerating system on the overall metabolism of ATP, the membranes were incubated with [^{14}C]ATP in the absence and presence of creatine phosphate and creatine kinase. A control containing [^{14}C]-ATP but no membranes was also analyzed. The results in Table II show that the control ATP was 99.4 % pure. Without an ATP regenerating system over 80 % of the added ATP is converted to other products, these being ADP, AMP, cyclic AMP and adenosine. The ATPase, and 5'-nucleotidase activities are much higher than the adenylate cyclase activity. The ATP regenerating system was effective in maintaining a high level of ATP and a low level of ADP but an appreciable amount of adenosine still formed. The effect of the ATP regenerating system on adenylate cyclase was negligible under these experimental conditions. Since the ATP level was main-

TABLE I

EFFECT OF DETERGENTS ON THE ACTIVITIES OF PLASMA MEMBRANE ENZYMES

Detergent (0.1%)	*Enzyme activity**	
	ATPase	*5'-Nucleotidase*
None (control)	9.4 ± 1.9	14.5 ± 0.6
Sodium dodecyl sulfate	2.6 ± 1.6	1.2 ± 0.7
Deoxycholate	11.1 ± 2.4	18.2 ± 2.2
Triton	9.1 ± 1.4	20.8 ± 2.5
Lubrol	12.3 ± 2.8	16.4 ± 2.5

* μmoles of P_i/mg membrane protein per 20 min at 37 °C. Mean ± S.E. of 3 experiments. Membranes were made by the method of Ray[21].

TABLE II

EFFECT OF AN ATP REGENERATING SYSTEM ON ATP METABOLISM IN ISOLATED PLASMA MEMBRANES

Membranes were made by the method of Ray[21]. The adenine nucleotides and adenosine were separated by chromatography as explained in the text. The adenylate cyclase system is given in the text. The ATP regenerating system contained creatine kinase (2.5 units) and phosphocreatine (10 mM).

	Percent of total cpm		
	No regenerating system	*With regenerating system*	*Control no membrane*
ATP	18.4	76.7	99.4
ADP	40.9	4.4	0.41
AMP	16.1	1.3	0.04
cyclic AMP	0.1	0.1	0.05
Adenosine	24.1	17.1	0.05

tained at a high level but the amount of adenosine produced was still appreciable, it appears that the ATP pyrophosphohydrolase activity in this system was appreciable. This enzyme yields 5′-AMP which is subsequently converted to adenosine by 5′-nucleotidase. The overall metabolic pathways involving ATP metabolism in the membrane are given in Fig. 7. The complexity of the reactions demonstrates the unique characteristics of adenine nucleotide metabolism in the membrane and points out the uncertainty in measuring the enzyme activities since cyclic processes are involved. The assay for ATPase may be in error if one measures only the P_i liberated since the P_i can also be generated by ADPase and 5′-nucleotidase. If the ATPase, ATP pyrophosphohydrolase and 5′-nucleotidase activities are considered, the net result of these three enzymes is to form adenosine, pyrophosphate and P_i as major end products. The data in Table II and Fig. 6 show that adenosine is a major product when membranes are incubated with [^{14}C]ATP with or without an ATP regenerating system.

In our previous work we failed to observe a F^- stimulation of the rat liver plasma membrane bound adenylate cyclase[25] whereas others[26–29] have observed the F^- stimulation of this enzyme in several different membrane preparations. We therefore investigated the F^- and sodium dodecyl sulfate effect on adenylate cyclase in membranes made by the method of Ray[21] and Neville[22] and in a crude membrane preparation of rat liver. The results are given in Table III. It can be seen that F^- and sodium dodecyl sulfate gave a marked stimulation of adenylate cyclase in membranes prepared by the method of Neville. F^- and sodium dodecyl sulfate also gave an appreciable stimulation of adenylate cyclase in the crude membrane fraction (the first low speed precipitate in the Ray preparation). F^- gave no stimulation of adenylate cyclase in membranes prepared by the method of Ray but sodium dodecyl sulfate did stimulate the enzyme. When membranes prepared by the method of Ray were washed with EDTA to remove bound Ca^{2+} (the method of Ray uses 0.5 mM Ca^{2+}

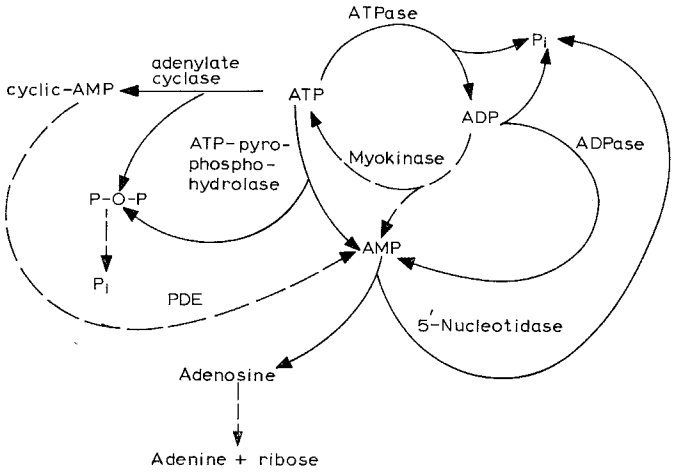

Fig. 7. Pathways for ATP metabolism in isolated plasma membranes. The dashed lines represent minor pathways or pathways not yet known. The solid lines represent known pathways. PDE = phosphodiesterase (EC 3.1.4.1), ATP-pyrophosphohydrolase (EC 3.6.1.8), ADPase (EC 3.6.1.6), myokinase (EC 2.7.4.3) and adenylate cyclase.

in the medium for preparing the membranes) the F^- stimulation was not observed primarily because the basal adenylate cyclase activity was markedly increased.

With membranes prepared by the method of Neville and in the crude membrane fraction of the method of Ray the F^- and sodium dodecyl sulfate effects were not additive.

TABLE III

THE EFFECT OF NaF AND SODIUM DODECYL SULFATE ON ADENYLATE CYCLASE ACTIVITY

NaF was tested at 10 mM concentration and sodium dodecyl sulfate at 0.1% concentration. The results are the mean ± S.E. of 3 experiments. The enzyme activity is expressed as nmoles of cyclic AMP formed per mg membrane protein per 15 min. The dash signifies not tested.

System	*Enzyme activity*			
	Control	*NaF*	*Sodium dodecyl sulfate*	*NaF + sodium dodecyl sulfate*
LSS-membrane fraction *	0.78 ± 0.16	2.36 ± 0.61	3.67 ± 1.3	3.25 ± 0.71
Membranes (Neville) **	0.33 ± 0.12	1.54 ± 0.53	2.2 ± 1.1	2.84 ± 0.28
Membranes (Ray) ***	0.32 ± 0.13	0.12 ± 0.06	1.76 ± 0.49	—
Membranes (Ray), EDTA washed	3.98 ± 1.4	3.0 ± 0.79	—	—

* This fraction is the low speed spin (LSS) (2000 rev./min) of the homogenate used in preparing membranes by the method of Ray[21]. This fraction consists mainly of plasma membranes and nuclei.

** Partially purified membranes made by the method of Neville[22].

*** Membranes made by the method of Ray[21].

DISCUSSION

The present studies demonstrate that different types of detergents have markedly different effects on the structure of the plasma membrane and on the activity of membrane bound enzymes. It is also shown that ATP metabolism in the membrane is complex and makes the assay for certain enzymes acting on adenine nucleotides rather empirical. For example the assay for ATPase activity by measuring only the P_i released may not be a true measure of this enzyme and can give erroneous results since P_i can also be produced by 5′-nucleotidase and ADPase. The assay for the enzymes is complicated by cyclic processes (Fig. 7) which regenerate common intermediates or starting compound. The use of [γ-^{32}P]ATP as substrate may circumvent some of these problems. In any case a major end product of the various enzymes acting on ATP, ADP and AMP is adenosine.

Previous work has indicated that ATPase[18,19] and 5′-nucleotidase[10] are lipoprotein enzymes and that both lipid and protein are necessary for full activity. Since sodium dodecyl sulfate and deoxycholate have been shown to alter lipoproteins of the rat liver membrane[11,12] these detergents were tested for their ability to solubilize the plasma membrane and their effect on the membrane-bound enzymes. The results show that a simple correlation between these two parameters does not exist. Thus sodium dodecyl sulfate and deoxycholate both solubilize the membrane but sodium dodecyl sulfate inhibits ATPase and 5′-nucleotidase whereas deoxycholate slightly stimulates these enzymes. Moreover, sodium dodecyl sulfate stimulates greatly the adenylate cyclase activity. One may speculate that the sodium dodecyl sulfate stimula-

tion of adenylate cyclase is due to its inhibition of ATPase which maintains a higher level of ATP and thereby stimulates adenylate cyclase. This explanation is not favored by studies in which an ATP regenerating system was added to the membrane system. The ATP regenerating did maintain a high level of ATP but had no effect on the adenylate cyclase activity.

Sutherland *et al.*[26] using Triton and Levey[30] using Lubrol, found that these detergents did not inhibit adenylate cyclase activity. Birnbaumer *et al.*[31] have recently studied the effects of detergents on adenylate cyclase of the fat cell. Their data show that 0.04–0.16 % digitonin caused a slight stimulation of basal adenylate cyclase activity, gave a marked enhancement of the F^- stimulation, but inhibited the hormone stimulation. These workers state (no data given) that 0.008 % sodium dodecyl sulfate stimulated the F^- response in liver adenylate cyclase system by 2-fold and gave a 40 % loss of the glucagon response. They further state that Triton X-100 and deoxycholate inhibit the response to both F^- and glucagon in a similar fashion.

The mechanism of hormone, F^-, and detergent stimulation of adenylate cyclase is unknown. It seems likely from the work of others[31–35] that the hormones act at a different site than does F^-. The effects of hormones are seen at very low concentrations (10^{-6}–10^{-9} M) whereas the effects of F^- and detergents are seen at a relatively high concentration (10^{-3}–10^{-4} M).

Hormone binding is most likely related to the hormone stimulation of adenylate cyclase. This concept led us to study the binding of glucagon and epinephrine to the plasma membrane[25,36]. The binding of the hormone is believed to initiate a change which is transmitted to the enzyme. It is unlikely that the hormone acts directly on an adenylate cyclase which consists only of one polypeptide chain (*i.e.* one subunit). This would make it difficult to explain the effects of various hormones and agents on the adenylate cyclase. It seems more plausible to invoke a mechanism similar to that recently observed for the activation of the protein kinase by cyclic AMP[37–39].

Ca^{2+} are known to bind to membranes and to "tighten" the membrane structure[40]. Apparently adenylate cyclase in the calcium stabilized membrane may be refractory to F^-. Calcium ion forms a non-dissociable salt with F^-. However, this cannot explain the Ca^{2+} inhibition of the F^- effect since F^- was added in large excess. It appears that F^- is not capable of loosening up the calcium "tightened" membrane. However, sodium dodecyl sulfate stimulates adenylate cyclase in membranes prepared with and without Ca^{2+}. Sodium dodecyl sulfate may therefore disrupt both membranes and thereby exert its effect on adenylate cyclase.

A recent paper by Emmelot and Bos[41] has questioned the efficiency of our previous two-dimensional chromatography[25] for the assay of cyclic AMP, inferring that labeled adenine might interfere with cyclic AMP. In the present method we have modified the chromatographic system by substituting isopropanol–formic acid–water (70:5:30, v/v) for isopropanol–concentrated HCl–water (65:16.7:18.3, v/v) as the second solvent. This modification gives a complete separation of cyclic AMP from inosine, adenine, adenosine, hypoxanthine, 5'-AMP, ADP and ATP. The activities for adenylate cyclase which we obtain by the modified system are of the same magnitude as that obtained by Pohl *et al.*[32] and Emmelot and Bos[42]. In the former system[25] we did obtain a good separation of cyclic AMP from ATP, ADP, 5'-AMP, adenosine and hypoxanthine. Although we previously did not include adenine in the mixture of cold nucleotides, nucleosides and base, this has now been done. Adenine

is separated from cyclic AMP in this system. Possibly the strong HCl in the second solvent gave rise to a degradation product which interfered with cyclic AMP and that this product is removed by the butanol-water run as used by Emmelot and Bos. It should be emphasized that over-loading chromatograms can give incomplete separation of certain components which are separated by short distances. In our hands between 5–15 μg of each nucleotide, nucleoside or base added to the sample could be adequately separated by the new solvent system.

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